A glucose electrode using high-stability glucose-oxidase collagen membranes

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A very sensitive glucose electrode was developed using a glucose-oxidase membrane and an anodically polarized platinum disk. Calibration curves were linear over 4.5 concentration decades. It was adapted for human blood samples. The lifetime of glucose-oxidase collagen membranes was greater than 3 yr at 4°C and was ~6 mo at 20–30°C. DIABETES CARE 5: 203–206, MAY–JUNE 1982.

Among the various techniques available for designing a glucose sensor, the glucose electrode, which combines a glucose-oxidase membrane and an electrochemical detector, is certainly the most developed. Since the first report by Clark et al.[1] and Updike et al.,[2] different detectors have been tested and different methods of immobilization have been studied. These include entrapment of enzyme solution by semipermeable membrane or by gel, and coreticulation or covalent coupling.[3,4] However, one of the main problems encountered with such enzyme electrodes has been the instability of enzymatic membranes. We have developed a sensor[5–7] using glucose oxidase covalently bound to collagen by the acyl-azide procedure,[8–10] which has excellent stability.[11] This article summarizes the main analytical characteristics of such a sensor, especially with blood samples, and presents the results of stability studies in storage and operating conditions.

MATERIAL AND METHODS

Glucose electrode. Glucose oxidase was coupled by the previously described acyl-azide procedure applied to insoluble films of highly polymerized reconstituted collagen[8–10] prepared by the Centre Technique du Cuir (Lyon, France). Carboxyl groups were first esterified by immersion in a methanol-hydrochloride acid solution and then treated by hydrazine before reacting with nitrous acid. The acyl-azide groups of such activated collagen reacted spontaneously with a glucose-oxidase buffered solution.

As pointed out previously, we preferred to use the glucose-oxidase membrane in association with anodic detection of hydrogen peroxide so that the glucose electrode is less dependent on oxygen concentration and so that its output current increases with the glucose level.[4] Since such detection is not very selective to enzymatically generated hydrogen peroxide, we included in our device a compensating electrode with a nonenzymatic collagen membrane. Except for data processing, material and methods were identical to those previously described.[5–7]

Electrode reponses. When a glucose-containing sample is added to a solution of 0.2 M acetate buffer ± 0.1 M KCl (pH 5.7) into which both electrodes are dipped, three different current-versus-time curves may be recorded:

(1) I_2 is the output current of the nonenzymatic, compensating electrode, E_2. I_2 is the background response and is usually very low except if the sample contains electrochemical reducing species such as ascorbate, urate, or sulfites.

(2) $(I_1 - k \cdot I_2)$, where k is close to 1, and corresponds to the detection of enzymatically generated hydrogen peroxide; it reaches a steady-state value after 2–3 min; this is the steady-state response of the sensor.

(3) $d(I_1 - k \cdot I_2)/dt$ is maximum after 30–50 s; the height of this peak is the dynamic response of the sensor.

Data processing. In order to avoid time-consuming analyses of recordings, we added to the previous equipment a Hewlett-Packard 97S programmable table calculator. Its binary coded decimal inputs and outputs were interfaced to the potentiostat and to an electronic buret through a Solea-Tacussel Ionomate 80 digital millivoltmeter (Figure 1). We developed a program that performs two main functions: (1) determination of steady-state response by detecting stable output currents before and after the sample addition, and (2) control of glucose standard additions by the electronic buret. We found that such a device gave printed results and a precision that was equal to graphical analysis of analogue recordings, providing we fixed low enough stability criteria.

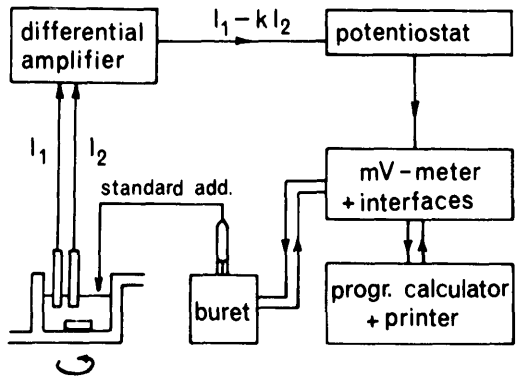

FIG. 1. Block diagram of electrodes and electronics for an automated in vitro glucose electrode.

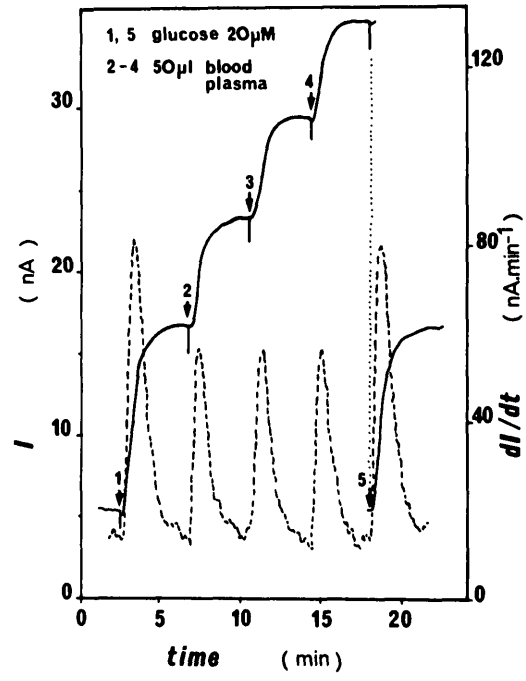

FIG. 2. Glucose determination in blood samples using steady state(—) or dynamic responses(--). Output current I was compensated, i.e., represents ($I_1 - k \cdot I_2$).

RESULTS

Analytical patterns of the glucose electrode. The following analytical patterns were obtained for in vitro glucose determinations: (1) Detection limit reached 10 nM or 10 pmol. (2) Glucose concentration was determined when it ranged between 10 nM and 10 mM. Steady-state and dynamic responses were proportional to glucose concentration over 4.5 decades, i.e., from 100 nM to 3 mM. (3) Response time was 30--50 s and 2–3 min for dynamic and steady-state signals, respectively. Cell and electrode washing time took an additional 2–3 min. (4) Precision, as estimated by standard deviation for 15 assays, reached 2–5% when glucose concentration ranged between 300 nM and 1 mM. (5) When using the differential device, i.e., a compensating electrode, selectivity coefficient was $\sim 5 \times 10^{-4}$ for usual metabolites (fructose, lactose, sucrose) and $\sim 5 \times 10^{-3}$ for electrochemical interferences (hydrogen peroxide, ascorbate, urate). (6) Glucose determinations were performed at temperatures ranging between 15°C and 40°C. Temperature coefficient of responses reached 6–11% and 4–5%/°C at 20°C and 30°C, respectively.

Blood glucose determination. The glucose electrode can be used with deproteinized or nondeproteinized samples of whole blood or with serum or plasma.[5,7] When 50–100 µl of these samples was added to the 20-ml buffer solution, the nonenzymatic current I_2 increased by 5–10 nA, whereas I_1 increased by 15–60 nA, depending on the sample. This result demonstrates the importance of compensating electrochemical interferences and adjusting coefficient k in ($I_1 - k \cdot I_2$) determinations by the differential current amplifier. This adjustment was actually tested by checking the balance of the steady-state responses of both electrodes to additions of electrochemical interferences such as hydrogen peroxide.

Figure 2 presents typical glucose assays in samples of nondeproteinized blood plasma. To check possible inhibitory effects on the glucose electrode responses, we usually performed pre- and posttest calibrations with aqueous glucose standards.

Thirty-two different deproteinized whole blood samples were analyzed for glucose. The correlation of the values obtained from steady-state and dynamic responses with those of a standard clinical method, i.e., the GOD-Perid Boeringher method, gave r^2 values of 0.95 and linear regression slopes equal to 1.053 and 0.998, respectively.[7]

Stability assays of glucose-oxidase collagen membranes. In a previous study, we found that the surface enzyme activities of glucose-oxidase membranes, which ranged from 8 to 80 mU/cm² in 0.1 M glucose, gave steady-state responses proportional to these activities.[6] Thus, the sole calibration of this glucose electrode, i.e., ΔI/ΔC, is a good way to monitor the surface activity of such glucose-oxidase membranes.

Initially, all membranes were stored at 4°C before or between tests at 30°C. Figure 3 shows the evolution of their respective steady-state responses during their operation time at 30°C. Only two of the seven membranes tested had a significant decrease in response within 20–30 h operation. We could not correlate the initial values of surface activities to such behavior. In most of the experiments, we used glucose-oxidase membranes giving rise to ΔI/ΔC values ranging from 2 to 4 mA/M, i.e., with surface activities ranging from 40 to 80 mU/cm²[6]. These membranes gave stable responses over several months of retesting, accumulating at least 30-h operation.

In a next step, we decided to store these glucose-oxidase collagen membranes at room temperature on the polarized electrode E_1 in order to simulate an instrument ready for use. The preparation for a glucose assay consisted only of temperature regulation of the cell at 30°C. We discovered that such storage conditions do not seem to change the surface activity of the membrane, as shown by the dark circles of Fig-

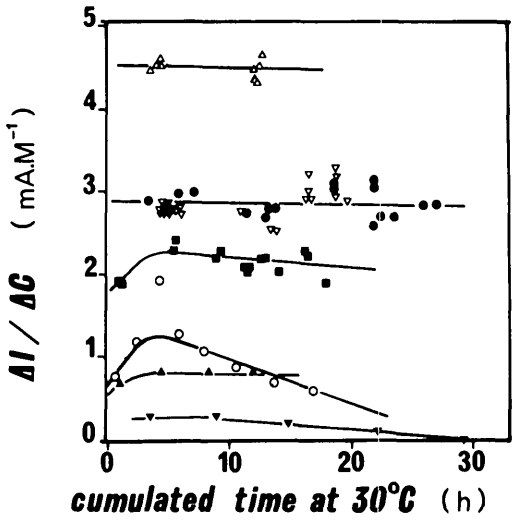

FIG. 3. Stability of steady-state responses during operation with seven different glucose-oxidase collagen membranes. All membranes were tested at 30°C and stored at 4°C, except (●), which was stored at room temperature.

ure 3. Thus we modified our storage procedure so that each freshly coupled membrane was stored at 4°C until it was first mounted on an electrode body; then it was kept at room temperature for several months, pressed against a polarized platinum disk, and used for more than 1000 assays at 30°C, accumulating 1000 h of operation. The evolution of daily or weekly calibrations for three membranes that had undergone various periods of storage at 4°C is presented in Figure 4. Two observations can be made from these results. First, the responses obtained with the same membrane may vary significantly over weeks or months. This does not seem to be related to poor regulation of the cell temperature, but more probably results from slight variations of geometrical parameters of the electrodes such as platinum disk-membrane distance. Indeed, it was found that an increase of the pressure of this enzymatic membrane against the platinum disk yields an important increase of steady-state responses and an even more important increase in dynamic responses.[12] Second, the responses do not decrease significantly after more than 2 yr at 4°C and 100 or even 200 days at 20–30°C; for such membranes, the surface activity of which ranged between 40 and 60 mU/cm², we did not find any correlation between the loss of activity of the enzymatic membranes and their operation time in contact with glucose-containing solutions, such as blood, food, or river water samples.[12]

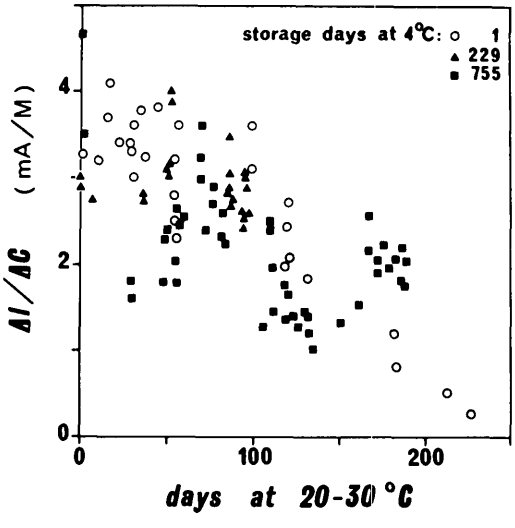

FIG. 4. Stability of steady-state responses when after (○) 1, (▲) 229, and (■) 755 days at 4°C. The glucose-oxidase collagen membranes were stored at room temperature and operated at 30°C (■). Data were obtained from ref. 13.

DISCUSSION AND CONCLUSION

It is generally claimed that enzyme immobilization, and especially its covalent coupling to an insoluble carrier, stabilizes the enzyme activity. Klibanov[14] recently reviewed the different factors to which the apparent stability is related.

When the enzymatic load is large enough and thus its intrinsic activity high compared with mass transfer kinetics, the rate-limiting factor is substrate diffusion and the intrinsic activity cannot be observed. Then, the overall reaction rate is minimally affected or not affected at all by a partial deactivation of the immobilized enzyme.[15] Several illustrations of such behavior have been given by Racine[16] or Comtat and Mahenc[17–19] using highly concentrated enzyme solutions entrapped by dialysis film, and by Scheller[20] or Gondo[21] using highly concentrated enzyme gels.

By contrast, the glucose-oxidase collagen membranes used in this glucose sensor seem to be kinetically limited by the enzymatic reaction.[6,22] A partial deactivation of the coupled glucose oxidase would directly affect the overall surface activity of the membrane and the electrode response. The results presented in this report together with those obtained with collagen-bound aspartate aminotransferase[11] show that even if diffusional effects can partially mask the intrinsic behavior of bound enzymes, the proteinaceous environment provided by collagen seems very favorable for storage and operational stability. Work is in progress to better define some geometric parameters of the glucose electrode, such as the thickness of the liquid film situated between the membrane and the platinum disk, used as a working electrode for hydrogen peroxide detection. Nevertheless, this glucose electrode can be used with daily calibration for several months at a time without replacing its high-stability glucose-oxidase collagen membrane.

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